Lead validation platforms

A battery of screening systems are under development to validate compounds for further development.

By definition, the pharmaceutical industry is based on the ability to successfully screen drug candidates. The approach of taking molecules from various sources and testing them initially in some kind of nonhuman system that mimics a human disease is a hallmark of modern drug discovery and the pharmaceutical industry's growth.

With the advent of such technologies as combinatorial chemistry, however, the traditional problems of lead generation no longer represent a bottleneck in drug development. The real issue now is not how many new compounds can one discover, but rather how many can one validate as potential therapeutic candidates for further development—on a daily basis.

This so-called lead validation problem is so daunting that technologies that accurately deliver a continue/discontinue prediction 70% of the time are deemed "very successful" and highly sought after. Which technologies will be able to break open this bottleneck in drug development?

Historical perspective

The ability to rapidly screen drug candidates has developed dramatically over the past 50 years. Screening targets for cancer is a good example of this evolution.

The US National Cancer Institute (NCI; Bethesda, MD) began formal screening efforts in 1955 to systematically find new anticancer compounds that had just begun to appear in early experiments[1]. The NCI began this program with mouse models of leukemia, sarcomas, and carcinomas. Yet after 20 years of experimentation, NCI researchers found that they had developed agents that cured cancer in mice but were largely ineffective in humans. From 1975 onward, NCI researchers refocused their testing strategy on xenograft mouse models. Human tumors were transplanted into immunosuppressed mice and compounds were tested on their ability to limit or kill these tumors.

The problem with this approach was that many of the key disease events, such as metastasis, did not occur in the xenograft models—limiting the predictive value of the approach. To overcome this limitation, the next step was to develop and test human cancer cell lines. The NCI currently has 60 human tumor lines, representing all major malignancies, which it uses to test lead compounds. This approach is currently also employed by many drug-development companies, but it must be remembered that these cell lines may not always reflect human cancers, because they are chosen based on their ability to propagate in vitro, outside the normal host environment. Nevertheless, the NCI's screening based on human cell lines has produced about 5,000 agents with antitumor activity[1] from screens of over 80,000 potential anticancer compounds. Of these 5,000 leads, an NCI database search that excludes those leads without novel mecha-

Table 1. Selected screening systems for lead validation

Company	Lead validation system
Acacia Biosciences (Richmond, CA)	Yeast-based genome reporter matrix
Affymax Research Institute (Palo Alto, CA)	Mammalian cell lines expressing high levels of transmembrane receptors used in scintillation proximity assays
Aurora Biosciences (San Diego, CA)	Fluorescent reporter systems for analysis of translocated cell lines
Axiom Biotechnologies (San Diego, CA)	Intracellular signaling profiles in human cell lines
BioDx (Pittsburgh, PA)	Human cell line fluorescence-based response assays
Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA)	Protease inhibitor screening by homogeneous time-resolved fluorescence assays
Caliper Technologies (Palo Alto, CA)	Chip-based assay systems
Chiron (Emeryville, CA)	Cytokine and chemokine receptor binding and kinetic assays
Evotec BioSystems (Hamburg, Germany)	Fluorescence correlation spectroscopy high-throughput assays
Exelixis Pharmaceuticals (S. San Francisco, CA)	Model genetic systems, *Drosophila* and *C. elegans*
Glaxo Wellcome (London)	Metamorphore imaging technologies
IGEN International (Gaithersburg, MD)	Electrochemiluminescence based assays of receptor ligand interactions
Ireti Quantum (La Jolla, CA)	Radio-frequency tagging
LifeSpan BioSciences (Seattle, WA)	High-throughput gene expression assays on libraries of human tissue cell lines
LJL BioSystems (Sunnyvale, CA)	Fluorescence polarization assays
Merck Research Laboratories (Rahway, NJ)	Proximity scintillation assays for enzyme/substrate and receptor/ligand interactions
Millennium Pharmaceuticals (Cambridge, MA)	Cell adhesion inhibition assays
Novalon Pharmaceutical (Chapel Hill, NC)	Peptide library competitive binding assays
Novartis Pharma (Basel, Switzerland)	Double-transfected cell lines monitoring the HIV-1 Rev/RRE interaction
NovaScreen (Hanover, MD)	Contract research; 120+ receptor binding assays
Oncogene Science (Uniondale, CA)	Yeast artificial chromosome-luciferase reporter assays
Pfizer (New York)	High-throughput phosphodiesterase assays
Pharmacopeia (Princeton, NJ)	Fluorescence-based enzyme assays in 1,536 well plates
Radioactive Insect Replacement CytoTechnologies (Belgium)	Bio- and chemiluminescence high-throughput toxicity assays
Rational Therapeutics (Long Beach, CA)	Cell-based apoptosis assays
Rhone-Poulenc Rorer (Collegeville, PA)	Cell-based reporter gene assays for transcription events
Scriptgen (Medford, MA)	Protein and nucleic acid molecular stability based assays
Small Molecule Therapeutics (now MorphoChem, Monmouth Junction, NJ)	Microbial and bioorganometallic screening systems
Syngatic Pharmaceutical (Paramus, NJ)	Cell lines expressing cloned human receptors
University of Massachusetts Medical Center (Boston, MA)	High-throughput nucleic acid structure probing
Xenova (Berkshire, UK)	Multiple label and time-resolved fluorescence assays for natural products screening
Zeneca Pharmaceuticals (London)	Homogeneous fluorescence assays (HTRF)

Source: Biovista (www.biovista.com)

nisms of action has selected 1,200 of these compounds for further testing.

Many high-throughput approaches today use automated one-to-one binding assays. In this approach, a target of interest, such as an enzyme known to be involved in a disease, is exposed to potential inhibitors in an enzyme-function test. While this approach makes sense *a priori*—and forms the cornerstone of nearly all current commercial validation programs—in actual practice its accuracy is constrained by the fundamental assumption that a good in vitro inhibitor of an enzyme will also work in the clinic. To overcome this problem, the next generation of high-throughput lead-validation platforms may be based on combinations of automated cellular assays, and cell-free "molecular drug response cascades."

Current state

The so-called clonogenic assay represents another aspect of the cellular assay. Here, the patient's own tumor cells are cultured in vitro, and potential drugs tested against them for ones that work specifically on that cancer. Comparisons of a specific agent's effect on a xenograft mouse model versus a clonogenic assay reveal significant differences. For example, colon cancer cells in xenografts respond to radiation and disappear largely because they are deficient in cell-cycle signaling checkpoint systems regulating regrowth, whereas a clonogenic assay of similar cells is unaffected by the checkpoint deficiency[2].

Companies are now developing dedicated cell-based assays in order to automate the process for more high-speed lead validation (see Table 1). These assays all attempt to more closely mimic a specific disease state than is possible through xenograft or clonogenic assays. For example, Rational Therapeutics has developed an ex vivo apoptosis (EVA) assay, which detects the differential morphology of tumor cells as they undergo cell death (apoptosis) in response to anti-cancer agents[3]. Another company, Synaptic Pharmaceutical inserts cloned human receptor genes into cell lines and screens for compounds that bind to target receptor subtypes[4]. Acacia Biosciences uses yeast cells to profile their response to lead compounds at the level of specific gene mutations, and also phenotypic responses[5]. Exelixis Pharmaceuticals uses whole model organisms whose genome is essentially known, such as the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, to test mutations and phenotypic responses as lead validation screens. Small Molecule Therapeutics (now MorphoChem) is developing microbial screens and assays based on the transmembrane receptor tyrosine kinase system.

In addition to these cell-line based assays, probing further refinement of the one-to-one intermolecular interactions approach is proving to be a key component of many ambitious screening programs. Most of these approaches employ technologies that allow more sensitive detection and quantification in a high-throughput format.

For example, confocal microscopy enables the detection of faint and/or small fluorescent signals by eliminating fluorescent light that is outside the plane of focus. Evotec BioSystems is incorporating a version of confocal microscopy called fluorescence correlation spectroscopy (FCS) into an automated ultrahigh-throughput screening system that the company calls EVOscreen. It enables the quantification of parameters such as fluorescence half-life, energy transfer, brightness, and spectral shift, which can be interpreted by customized software as molecular binding constants, polymerization of molecules, conformational changes in proteins, or cellular events such as transcription, signal transduction, or endocytosis.

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Industry challenges

The key lead validation challenge is the development of high-throughput screens that also provide clinically useful information. Inevitably, there is a tradeoff between the ease and speed of a test, and value of the information it gives. For screens based on intermolecular interactions where the target is either free in solution or immobilized on a surface, miniaturization and robotic liquid handling present a technical limitation. For these assays, the synthesis of appropriately labeled molecules for fluorescence or radiometric measurements is not always easy. Molecules derivatized in such ways often lose the specific biological activities that made them useful tools in the first place. Recombinant methods often help in such cases. For example, DNA topoisomerase has been engineered to contain a biotin segment that enabled the cloned fusion protein to be purified from crude cell extracts by streptavidin-coated scintillation proximity beads. This assay allowed the enzyme to be purified and also to be screened with inhibitor compounds[6]. Several dedicated instruments have been developed to handle nanoliter volumes in multi-well plate formats and also on chips. Companies that have developed such systems include Evotec, Cartesian Engineering (Durham, NC), Packard Instrument Company (Palo Alto, CA), Caliper Technologies, Aurora Biosciences, LJL BioSystems, and others.

Another challenge to the industry is that lead validation assays are limited by the intrinsic difficulties of the particular cell or organism models they depend on. Such assays are essentially determined by the nature of the biological event that the drug lead will play a role in. Sometimes the event is simulated in a cell model, and in this case the assay is limited by the inherent difficulties of the model. For example, cell death or apoptosis is a major biological event that correlates with cancer, where the latter actually inhibits normally occurring apoptosis. Here, the idea is to develop anti-cancer agents that induce apoptosis in cancer cells. Lead validation assays, therefore, focus on apoptosis-specific events, such as the expression of apoptosis-causing genes in cell models[7]. The limitation here is that these assays are transient transfection ones, where the apoptosis genes are induced transiently and often produce too much of the desired protein, thus invalidating the assay. There is, therefore, a constant effort to improve these assays and make them as physiologically relevant as possible.

The future

Advances in our understanding of molecular pathways and cascades within cells, such as signal transduction and apoptosis, which are at the heart of many disease conditions, promise to provide highly specific assays for disease states. Inappropriate signal transduction cascades, for example, are implicated in cancer, autoimmune, inflammatory, neurologic, and cardiovascular disorders. Reagents that enable the dissection of these cascades, such as highly specific antibodies and small molecule modulators, are becoming available from companies such as Upstate Biotechnology (Lake Placid, NY) and others. These reagents can be used together with standardized cell lines in automated high-throughput formats to validate potential leads in terms of specific effects on disease-specific molecular cascades. Whether cell-based or cell-free, such molecular drug response cascades are likely to offer information that is more clinically relevant than current high-throughput validation systems, and offer, therefore, significant promise toward solving or alleviating the [illegible] validation

tion issue of drug development.

In addition, lead validation assays will take more forms than at present and benefit from novel approaches. For example, the use of quantitative polymerase chain reaction (PCR) in animal models of leukemia can help predict the survival of the animals when treated with different drugs[8]. Here, quantitative PCR is used to measure tumor burden in the animals, which will decrease as a result of any therapeutic benefit from anti-cancer leads.

Finally, the increasing integration of lead validation assays into systematic lead development efforts will also become more commonplace. For example, DNA gyrase catalyzes the condensation of the DNA structure, and is therefore a significant target for novel drug discovery of anti-cancer agents, anti-microbials, and anti-virals. In a recent report, random screening of lead libraries failed to produce good lead inhibitors of the enzyme. However, the integration of in silico screening for novel small inhibitors, lead validation assays based on biased high-throughput DNA gyrase screens and biophysical methods, and a systematic lead optimization process has produced an inhibitor 10-fold more potent than the known inhibitor novobiocin[9].

Conclusions

With combinatorial chemistry libraries nearly ubiquitous in the drug-development community, millions of potential drug leads are being screened (see Combinatorial chemistry, pp. 50–52). The methods described here are aimed at providing critical data that will help decide which, if any, will be developed further as therapeutics. The central issue these methods must address is whether they provide information that is clinically relevant.

It will be a synthesis of the information derived from as many of these screening techniques as possible that will provide the most important insights into the behavior of a lead in a clinical setting.

For high-throughput screens based on the interaction of a target molecule with lead compounds in isolation, there is little clinical context to lend confidence to their results, unless they are highly specific in mimicking a disease state. At the other end of the scale, cell lines probed for genotypic and phenotypic responses incorporate more of a clinical context than two molecules in solution, but are not amenable at present to standardized high throughput.

The future is very exciting in this area, because of advances in instrumentation that enable the handling of very small quantities of cells and reagents, and also the development of various detection methods that report the occurrence of specific molecular events. It is most likely that it will be a synthesis of the information derived from as many of these screening techniques as possible that will provide the most important insights into the behavior of a lead in a clinical setting.

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